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Electronic Supporting Information

NanoArmoring: Strategies for Preparation of Multi-Catalytic Enzyme Polymer Conjugates and Enhancement of High Temperature Biocatalysis

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1. EXPERIMENTAL SECTION

1.1 Zeta Potential Studies. Zeta potential of unmodified enzyme, enzyme-PAA and MEC's are measured using Brookhaven zeta plus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY) by laser doppler velocimetry. In each experiment, 1.5 mL of sample is used and the enzyme concentration is maintained at 0.01 mM. Smoluchowski fit by the software and matching electrophoretic mobility technique is employed to acquire the zeta potential values in mV.

1.2 Agarose Gel Electrophoresis. Agarose gel electrophoresis is done using horizontal gel apparatus by Gibco Model 200, Life Technologies Inc., Grand Island NY. Molecular biology grade agarose (0.5% w/v) is heated in microwave in pH 7, 40 mM Tris acetate buffer and left to gel in apparatus. After the gel is formed, samples are mixed with loading buffer containing 50% v/v glycerol and 0.01% m/v bromophenol blue, loaded into the gel and electrophoresis is carried out for 40 min at 100 V. Then gel is stained using 10% v/v acetic acid and 0.02% m/v coomassie blue overnight, and then destained with 10% v/v acetic acid overnight, photographed and described.

1.3 Circular Dichroism (CD). Secondary structure of unmodified enzymes, enzyme-PAA and MEC's are evaluated using CD spectroscopy. Structure of enzymes is evaluated in the ultraviolet (UV) range (195 nm to 250 nm) using Jasco 710 spectropolarimeter. CD spectra of each sample are normalized for 1 μM of total enzyme concentration. Enzyme concentration is determined by UV visible spectroscopy before CD studies. The extinction coefficients used are: GOx (1.4×10^4 M^{-1} cm^{-1} at 450 nm)[1], HRP (102 mM^{-1} cm^{-1} at 403 nm)[2], Lip (175 mM^{-1} cm^{-1} at 280 nm)[3], AP (1.0×10^5 M^{-1} cm^{-1} at 280 nm)[4], LDH (182 mM^{-1} cm^{-1} at 410 nm). [5]

1.4 Transmission Electron Microscopy (TEM). Morphology of all the samples are analyzed using tecnai T 12 TEM functioning at an accelerating voltage of 120 kV and at step 3. Aqueous samples of glucose oxidase (GOx), GOx-polyacrylic acid (GOx-PAA) and 5-P are prepared at enzyme concentration of 5-10 nM. These samples are drop casted on TEM grids covered with fomvar film. Excess solution is removed by blotting. TEM grids bearing samples are then stained with 0.5 wt % of uranyl acetate, dried for 20 mins and imaged.

1.5 Dynamic Light Scattering (DLS). Size of the enzymes, enzyme-PAA and MEC's is evaluated using CoolBatch+ dynamic light scattering apparatus with Precision Detectors (PD2074 N) (Varian Inc.) used in combination with a 0.5 x 0.5 cm^2 square cuvette and 658 nm excitation laser source at 90º geometry. Samples corresponding to 0.5 nM of total enzymes in pH 7, PB are filtered through 0.2 -micron filter (PVDF, 13 mm, Fisher Scientific) and equilibrated for 300 s at 25 ˚C; 5 repetitions with 60 accumulations are recorded. Precision Elucidate Version 1.1.0.9 software is used to run the experiment and Deconvolve Version 5.5 is used to process the data. Average hydrodynamic radius of enzymes and enzyme-conjugates are computed based on triplicates measurements.

1.6 Catalytic activity measurements. Activity studies of unmodified enzymes, enzyme-PAA and MEC's (5-P, 5-S) are carried out using appropriate enzymatic assay methods. Catalytic activities for GOx are determined using appropriate assay methods for samples containing GOx, including GOx, GOx-PAA, 5-P and 5-S. Equivalent concentration of enzyme for each activity assay is maintained at 1 μM and appropriate substrates are added using buffered solvents (Tris acetate or PB). Detailed activity assay protocols are presented in following sections. In all experiments the error bars are computed based on triplicates carried out of each measurement of that experiment.

1.6.1 Glucose oxidase (GOx) activity. Activity of unmodified GOx, GOx-PAA, 5-P and 5-S are measured using a cascade system by employing GOx and HRP as enzymes.[6] GOx will catalyze glucose to gluconolactone and H_2O_2. HRP will utilize H_2O_2 to oxidize o-methoxyphenol to product, which is traced at λ_{max} of 470 nm using UV spectroscopy. Specific activities are measured using 1 μM of GOx and HRP samples (in unmodified native form or in conjugate form), 0.125 mM o-methoxyphenol, 0.3 mM glucose in 10 mM PB at pH 7.4. Activity studies are carried out for 1 min and first 20-40 s are used for calculating the initial rate. Initial rate of GOx in PB at 25°C is referenced at 100% and used to calculate all the specific activities relating to GOx based enzyme systems. While carrying out the activity studies due to oxidation of glucose by GOx, H_2O_2 is produced, as a result of oxidation of glucose by GOx might deactivate other enzymes. The role of peroxidase in removing hydrogen peroxide is important only when GOx activity is utilized, not when other enzyme activities are being monitored. Thus, the current approach can be used without GOx or with GOx in the mix, as desired.

1.6.2 Horseradish peroxidase (HRP) activity. Catalytic activities of unmodified HRP, HRP-PAA, 5-P and 5-S are computed using H_2O_2 and o-methoxyphenol.[7] To establish activities, equivalent of 1 μM HRP in all samples are added to the cuvette containing 0.375 mM o-methoxyphenol and 0.125 mM H_2O_2 in 10 mM PB pH 7.4. Oxidant product of o-methoxyphenol with λ_{max} at 470 nm is quantified using colorimetric methods. Activity studies are carried out for 1 min and initial 10-20 s are used to compute the initial reaction rate. The rate of HRP in PB at 25°C is referenced as 100% and used to calculate the specific activities of samples containing HRP.

1.6.3 Lipase (Lip) activity. The catalytic activity of lip is carried out using 4-nitrophenyl acetate (4-NPA) as a substrate based on reported protocols.[8] A stock solution is prepared using

63 mg of 4-NPA dissolved in 10 mL methanol. A 1 mL aliquot of 4-NPA of stock solution is dissolved in 100 mL DI. Final concentration of 4-NPA is maintained at 0.0175 mM in the cuvette while carrying out activity studies in 10 mM PB at pH 7.4. These experiments are carried out for 3 min and initial rate for 60-80 s is used for calculation of specific activity for 1 μM of HRP. Esterification of 4-NPA to 4-nitrophenol is monitored at 405 nm using UV spectrometer. This initial rate for Lip is used as reference (100%) to calculate the specific activities of all other samples containing Lip.

1.6.4 Acid phosphatase (AP) activity. The catalytic activity of AP is established using 4-nitrophenyl phosphate (4-NPP) as a substrate.[9] While performing the activity studies concentration of AP and 4-NPP is maintained at 1 μM and 5 mM in the cuvette, respectively. The sample is first stirred in 10 mM PB, pH 7.4, in the cuvette and then substrate is added. Enzymatic catalysis of 4-NPP to 4-nitrophenol is quantified at 405 nm using UV spectrometer. Activity studies are carried out for 3 minutes and initial rate for 60-80 s is used for calculation of specific activity. Initial catalytic rate for AP at 25 °C is referenced at 100%. Therefore, catalytic activities of other samples such as AP-PAA, 5-P and 5-S are computed keeping AP as a reference.

1.6.5 Lactate dehydrogenase (LDH) activity. The catalytic activity of LDH is monitored using NADH as a substrate in presence of sodium pyruvate using 10 mM, pH 8 Tris acetate buffer (Tris).[10] The concentration of LDH, NADH and sodium pyruvate is maintained at 0.1 μM, 0.067 mM and 0.45 mM, respectively, in the cuvette. The oxidation of NADH to NAD was monitored at 340 nm using UV spectrometer.

1.7 Activity studies at high temperature (65 ºC). The temperature-controlled catalytic activities are determined at 65 ºC using five unmodified enzymes (GOx, HRP, LDH, AP, Lip),

single enzyme-PAA conjugates and 5-P or 5-S conjugates with appropriate substrates, solvents and buffers using protocols similar to the ones described in Section 1.5. In all experiments the error bars are computed based on triplicates carried out of each measurement of that experiment.

1.8 Kinetic studies. To gather kinetic parameters such as K_M and V_{max}, Lineweaver Burk plots are used. Identical activity assays are used that assist in gathering activity studies information. In case of each enzyme and the corresponding enzyme-PAA and MEC conjugates varying substrate concentrations are used to construct Lineweaver Burk plots. In all experiments the error bars are computed based on triplicates carried out of each measurement of that experiment.

In case of GOx activity assay, glucose is used as a substrate and its concentration is varied in the range of 0.075 – 0.45 mM. o-methoxyphenol is used as a substrate for HRP activity assay and the concentration range used is in the range of 0.0163 – 0.097 mM. In case of Lip activity assay, 4-nitrophenylacetate is used as a substrate in the range of 0.007 – 0.012 mM. For LDH activity assay, NADH is used as a substrate in the range of 0.0075 – 0.15 mM. Lastly, in case of AP activity assay, 4-nitrophenylphosphate is used as a substrate in the range of 2.7 – 16.2 mM. All the kinetic parameters are extracted using Lineweaver Burk plots.

1.9 Cellular toxicity of MEC's and enzyme-PAA biocatalysts in human embryonic kidney HEK-293T cells. The in vitro compatibility of MEC's and enzyme-PAA biocatalysts are investigated by co-incubating with human embryonic kidney HEK-293T cell lines under standard growth conditions (37 °C, 5 % CO_2 and 95 % relative humidity). Briefly, 0.5 x 10^5 HEK-293T cells are seeded in each well of 24 well plates (Corning Inc.) in 500 μL of complete growth media, which consist of DMEM (Dulbecco's Modified Eagle Medium) supplemented with 9% fetal bovine serum.

The cells are allowed to attach to the bottom of the 24 well plate and then exposed to different concentrations of polyacrylic acid separately conjugated with GOx, HRP, AP, LDH as well as the MEC's synthesized in a step-wise manner (5-P) and one-pot method (5-S). After four hours of incubation, the compatibility of samples are measured using following procedures performed in triplicates.

A. Intracellular metabolism

The intracellular metabolism of HEK-293T cells co-incubated with samples is measured by quantifying the intracellular dehydrogenase activity using CCK-8 kit as reported previously.

B. Phase-contrast optical microscopy

The overall extracellular morphology of a cell is an important indicator of its health. The morphology of the rapidly growing cells co-incubated with samples are monitored for short-term (0 – 4 hours) and for medium-term (after 24 hours of co-incubation) using optical microscopy. In all experiments the error bars are computed based on triplicates carried out of each measurement of that experiment.

2. SUPPLEMENTARY FIGURES

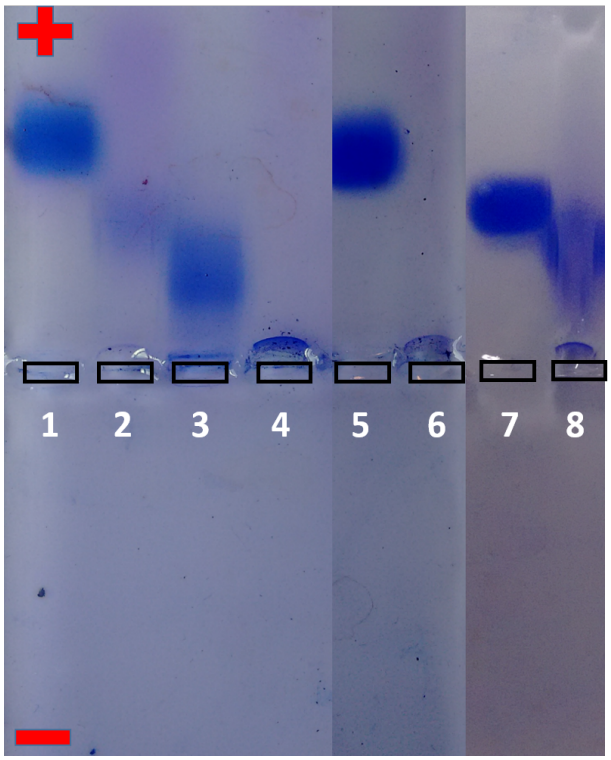

Figure S1: Agarose gel electrophoresis study of GOx, GOx-PAA, AP, AP-PAA, LDH, LDH-PAA, Lip and Lip-PAA from lane 1-8 consecutively. Agarose gel show successful conjugation of PAA to enzymes as enzyme-PAA conjugates got stuck in the wells or show less movement across the gel due to increase in size and extent of conjugation. Agarose gel is performed using 40 mM Tris acetate at pH 7.

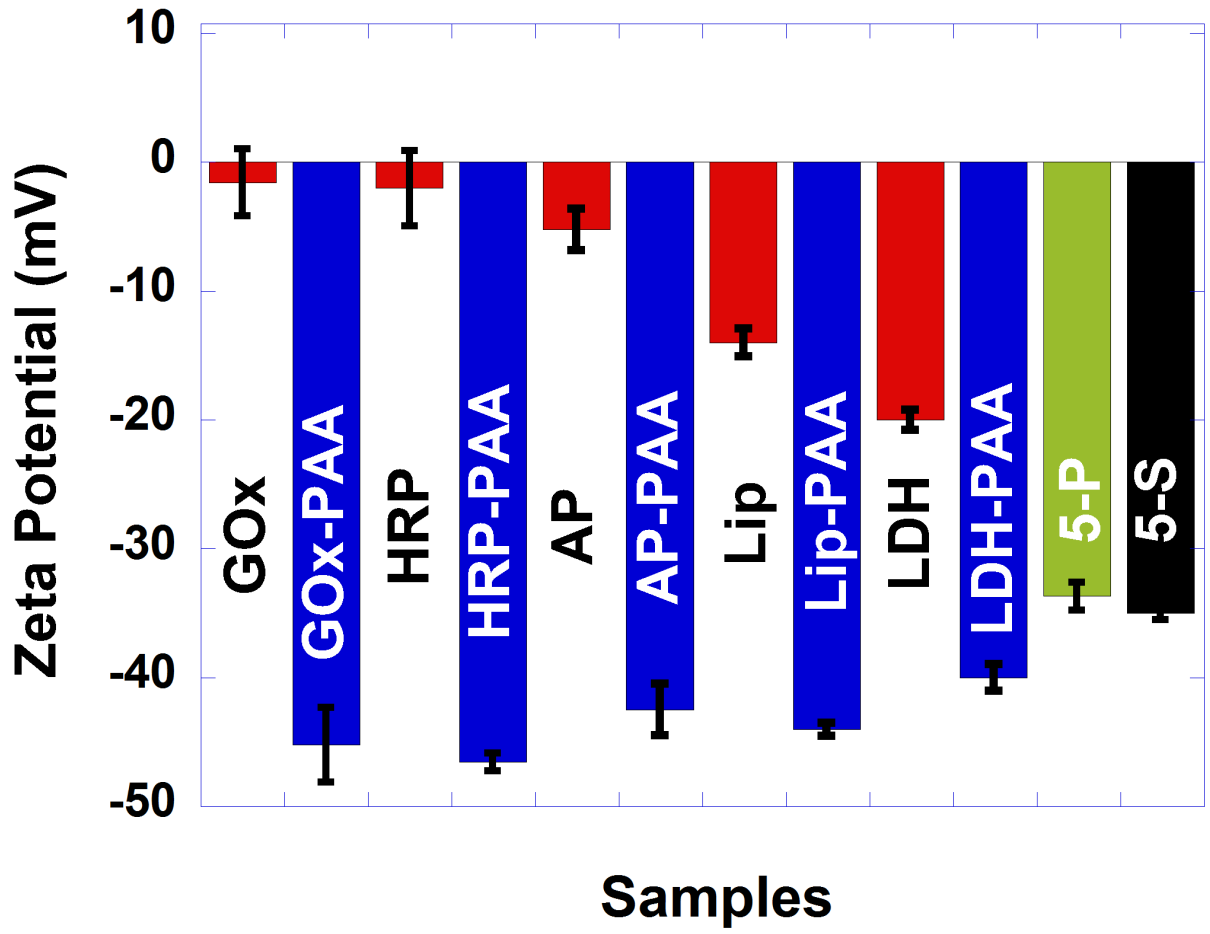

Figure S2: Zeta potential of unmodified enzymes, single enzyme-PAA and multienzyme complexes (MEC's) in pH 7.4 PB at 25 ºC. Zeta potential of GOx, GOx-PAAHRP, HRP-PAA, AP, AP-PAA, Lip, Lip-PAA, LDH, LDH-PAA, 5-P and 5-S are presented.

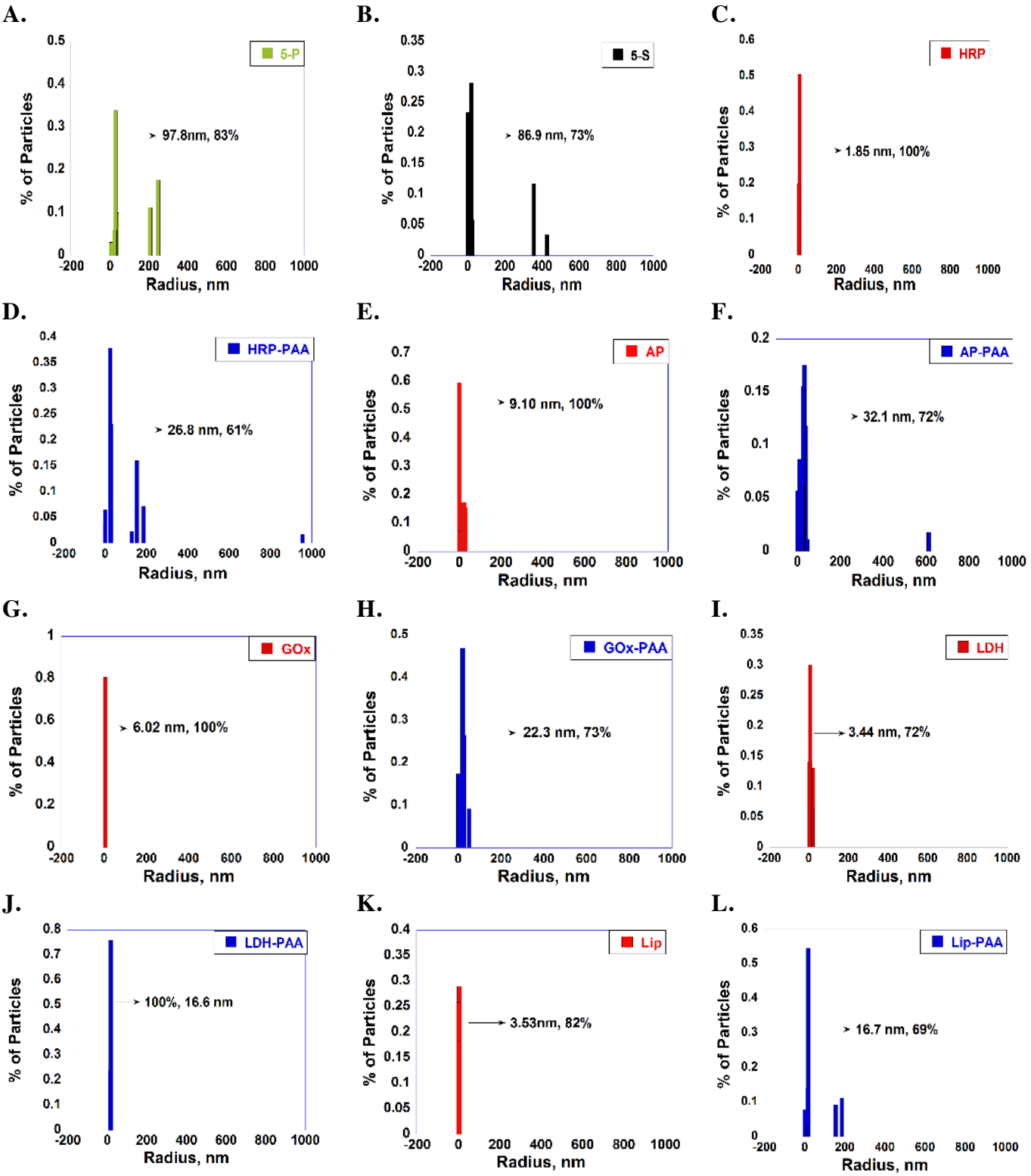

Figure S3: DLS spectra of all samples in 10 mM PB at pH 7. (A) 5-P, (B) 5-S, (C) HRP, (D) HRP-PAA, (E) AP, (F) AP-PAA, (G) GOx, (H) GOx-PAA, (I) LDH, (J) LDH-PAA, (K) Lip and (L) Lip-PAA.

Table S1. DLS data of MECs, enzyme-PAA and enzymes illustrating the size of the samples. The samples are analyzed at pH 7 PB at 25 ºC.

Sample	Hydrodynamic radius, nm	Sample	Hydrodynamic radius, nm
GOx	6.02	GOx-PAA	22.3
HRP	1.85	HRP-PAA	26.8
AP	9.10	AP-PAA	32.6
Lip	1.49	Lip-PAA	16.7
LDH	3.44	LDH-PAA	16.6
5-P	97.8	5-S	86.9

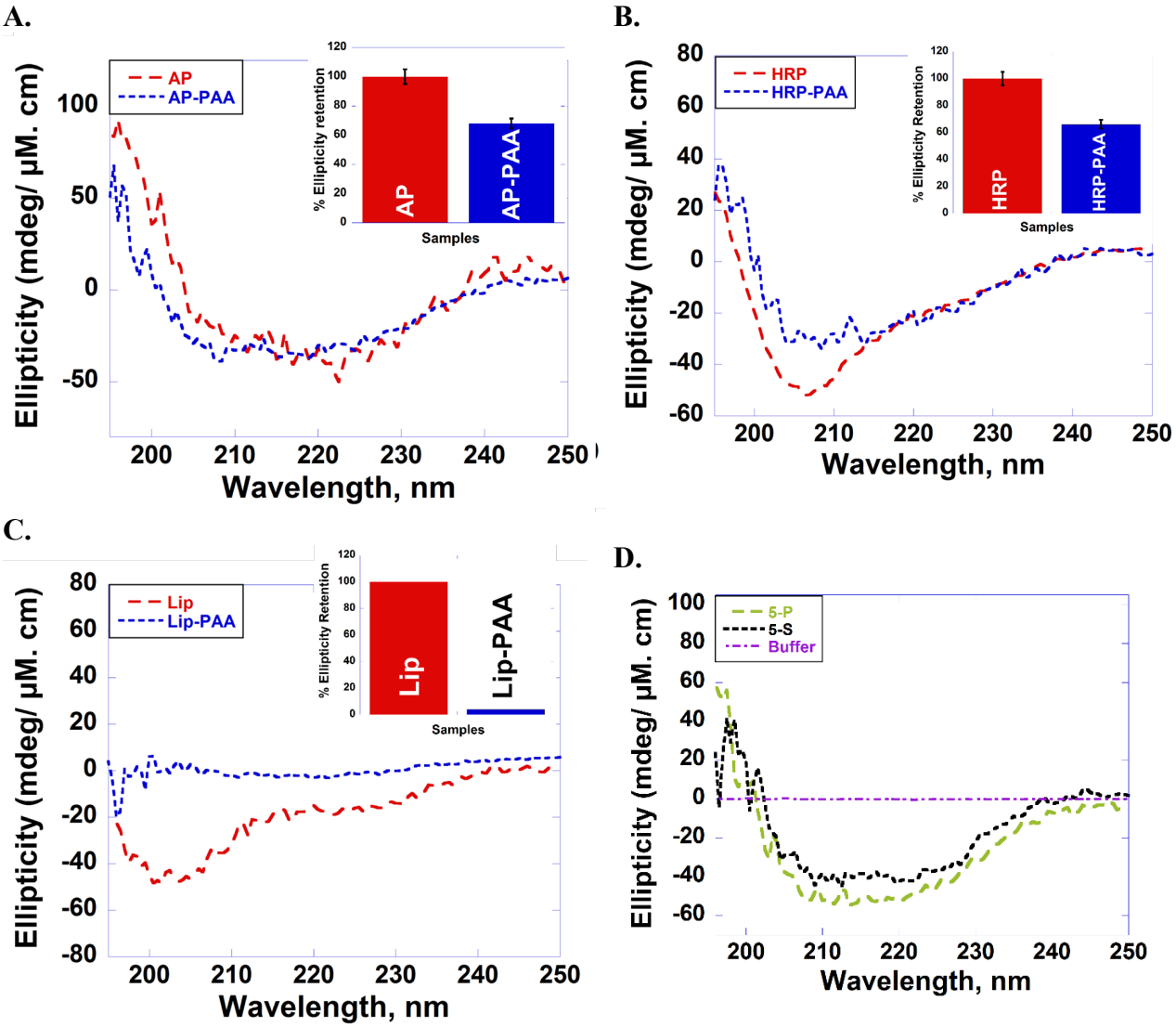

Figure S4: Circular dichroism (CD) spectra of (A) AP (red), AP-PAA (blue) and (B) HRP (red) HRP-PAA (blue) (C) Lip (red), Lip-PAA (blue) (D) 5-P (green) and 5-S (black). CD spectra are monitored from 190-250 nm in pH 7.4 PB at 25 ºC. The inset figure gives the relative ellipticity retention, when peak at 222 or 202 nm for unmodified enzyme is referenced at 100 %.

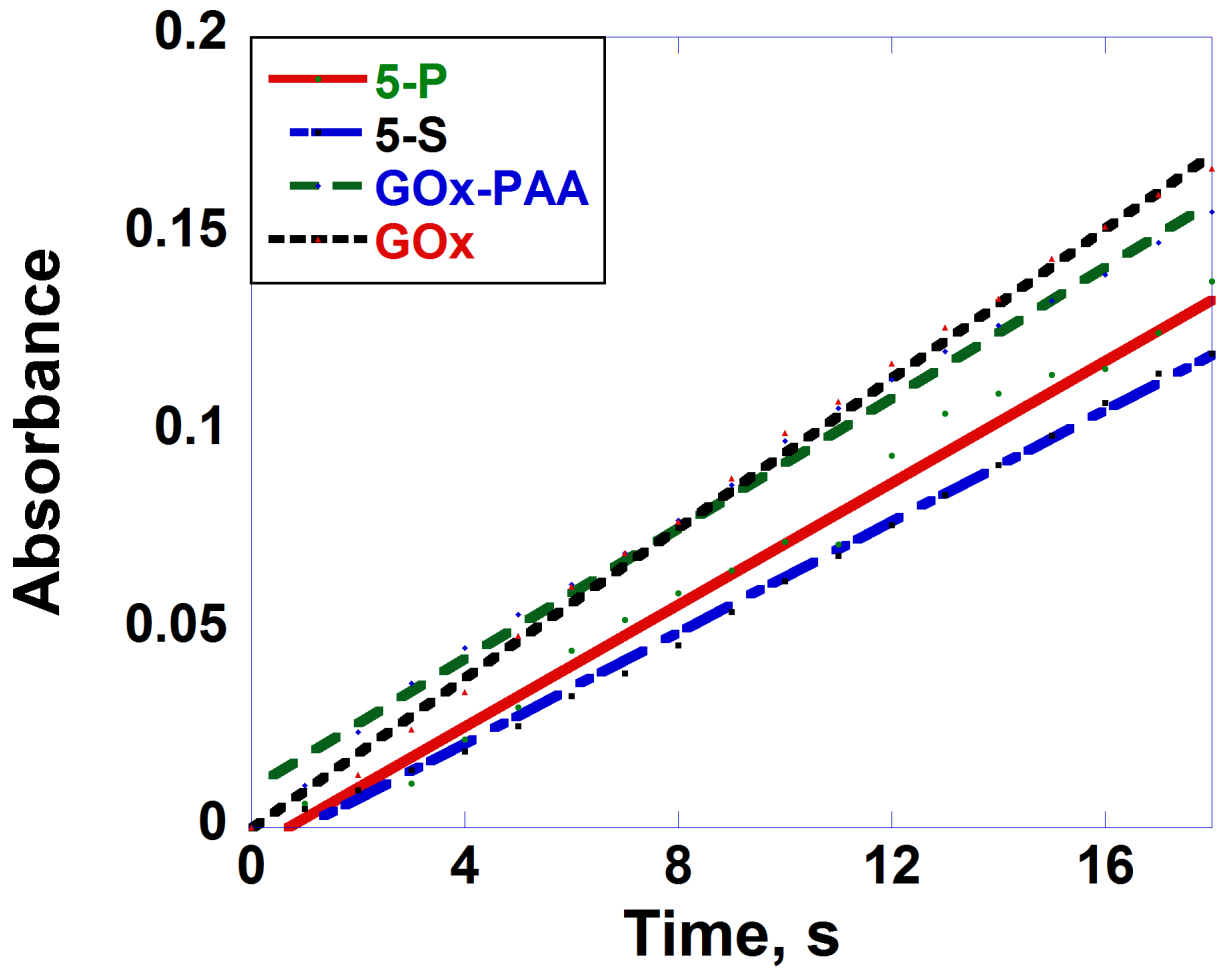

Figure S5: Activity study kinetics traces of 5-P, 5-S, GOx-PAA and GOx samples using glucose as a substrate. The activity of enzymes are calculated based on the slope of the first 20 seconds of kinetics of enzymatic conversion of glucose using GOx as an enzyme. Unmodified enzyme (GOx) is used as a reference at 100% and all the activities are calculated based on that for GOx activities. Similar strategy is followed for other enzyme activity assays. Activity studies were carried out in pH 7.4, 10 mM phosphate buffer at 25 ºC.

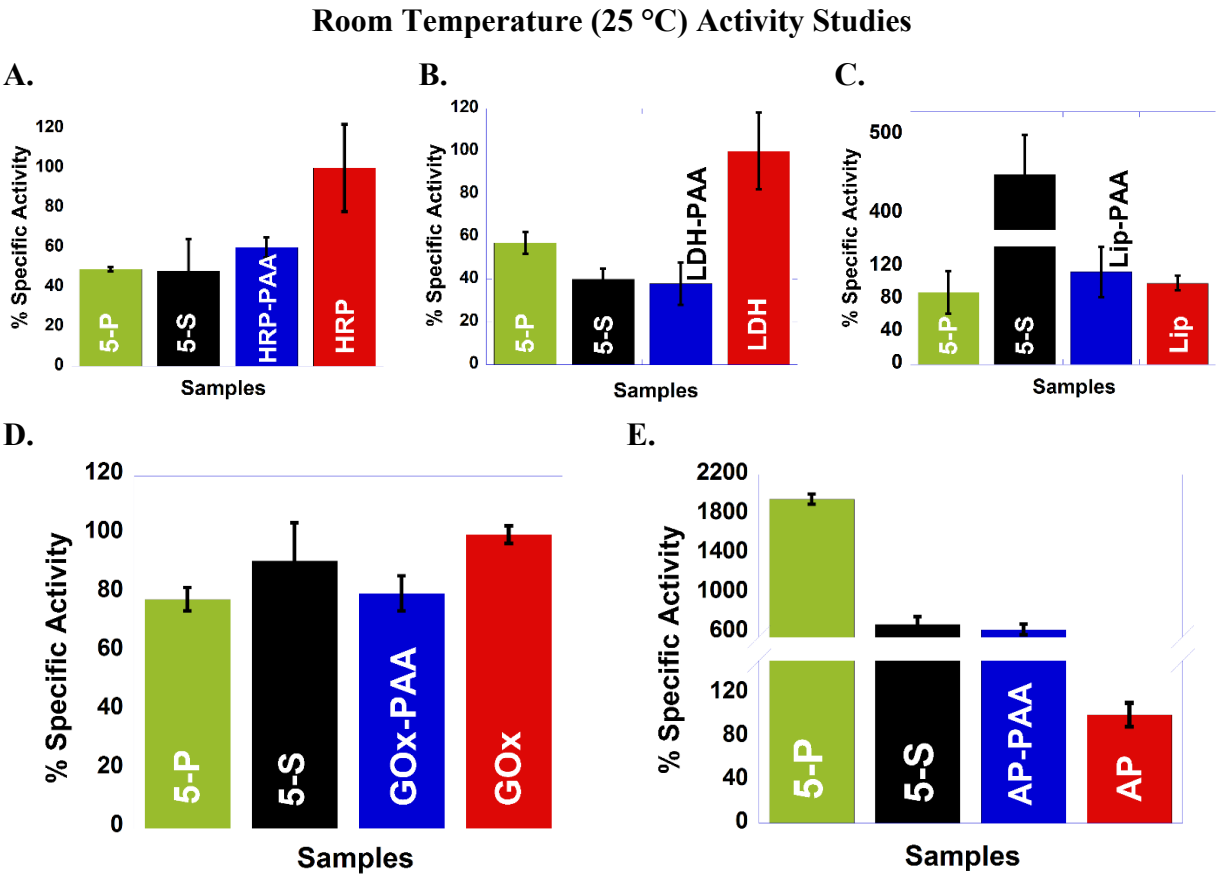

Figure S6: Activity studies were carried out at 25 °C in pH 7.4, 10 mM, PB for HRP, Lip, GOx, AP and 10 mM pH 8, Tris for LDH containing samples and data is presented. % specific activity is plotted on Y axis. To calculate % specific activity, initial rate of unmodified enzyme is referenced at 100% and all other samples are referenced relatively. In above figure % specific activity of HRP containing samples (A), LDH containing samples (B) and Lip containing samples (C) are presented. 5-P, 5-S are represented as green and black. HRP, HRP-PAA, LDH, LDH-PAA, Lip and Lip-PAA are represented as red, blue, red, blue, red and blue respectively.

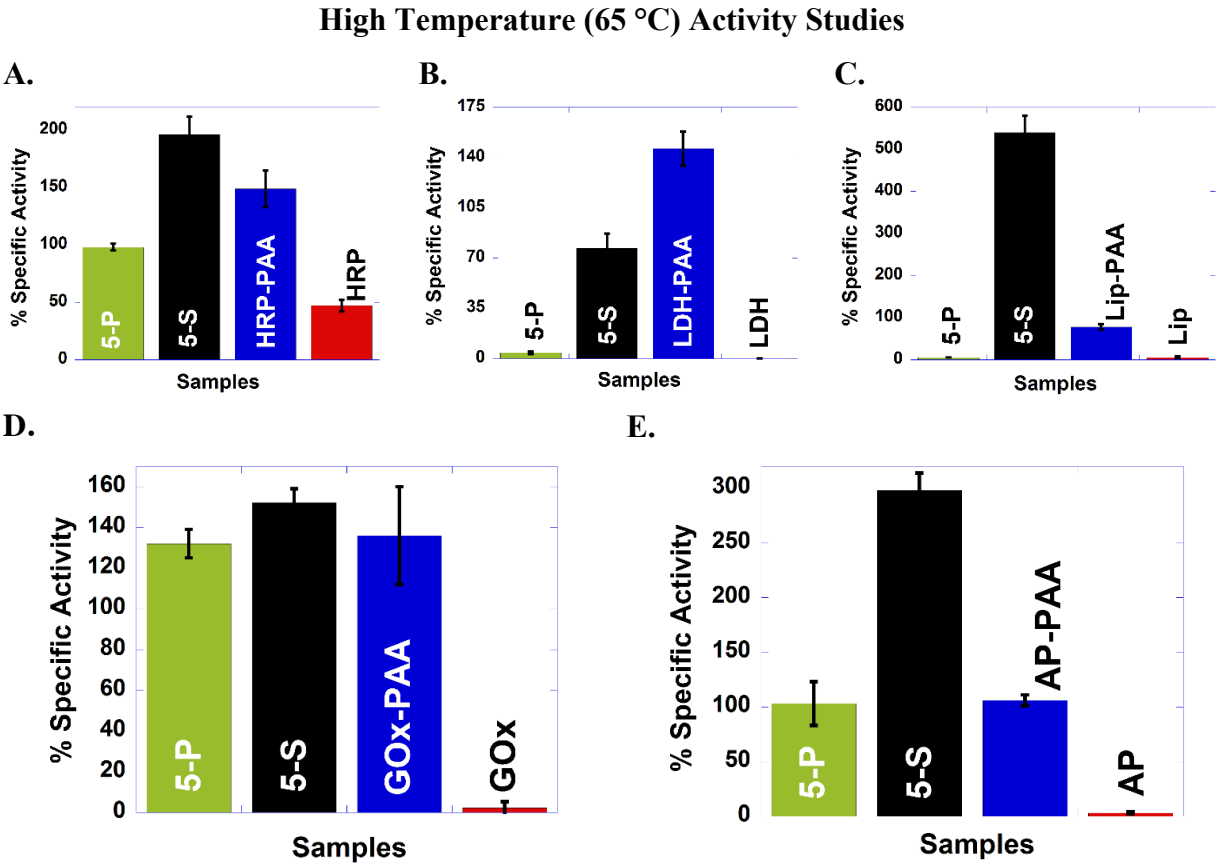

Figure S7: Activity studies are carried out at 65 °C in pH 7.4, 10 mM, PB for HRP, Lip, GOx, AP and 10 mM pH 8, Tris for LDH containing samples and data is presented. % specific activity is plotted on Y axis. To calculate % specific activity, initial rate of unmodified enzyme at 25 °C is referenced at 100% and all other samples are referenced relative to the unmodified enzymes. In above figure % specific activity of HRP (A), LDH (B) Lip (C) GOx (D) and AP (E) containing samples are presented. 5-P, 5-S are represented as green and black. All unmodified enzymes- HRP, LDH, Lip, GOx and AP are represented as red and enzyme-PAA conjugates- HRP-PAA, LDH-PAA, Lip-PAA, GOx-PAA and AP-PAA are represented as

blue.

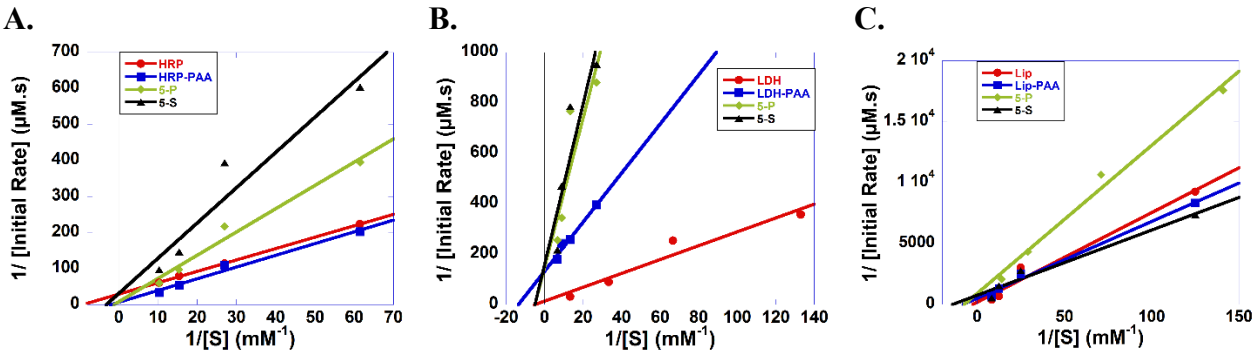

Figure S8: (A) Lineweaver burk plots of HRP (red), HRP-PAA (blue), 5-P (green) and 5-S (black) with increasing o-methoxyphenol (0.0163 – 0.097 mM). (B) Lineweaver Burk plots of LDH (red), LDH-PAA (blue), 5-P (green) and 5-S (black) with increasing NADH (0.007 – 0.15 mM). (C) Lineweaver Burk plots of Lip (red), Lip-PAA (blue), 5-P (green) and 5-S (black) with increasing 4-nitrophenylacetate (0.008 – 0.12 mM). Studies are carried out in pH 7.4, 10 mM PB (HRP, Lip) and pH 8, 10 mM Tris (LDH). All the enzyme concentrations, native and within conjugates, are 1 μM. All the data points are averages of triplicates of the measurement. Straight-line equations are fit to generate the data, as 1/ [initial rate] vs 1/[S] yields a straight line. The margin of error is < 1%.

Table S2. Kinetic parameters such K_M, V_{max}, K_{cat} and catalytic efficiency of all the enzymes, single enzyme-PAA, 5-P and 5-S. All the kinetic parameters are gathered in pH 7.4, 10 mM PB (GOx, AP, HRP and Lip) and pH 8, 10 mM Tris (LDH).

	K_M (mM)	V_{max} ($\mu M^{-1}s^{-1}$)	K_{cat} (s^{-1})	Catalytic Efficiency
GOx enzymatic assay				
GOx	5.17	0.533	0.533	0.103
GOx-PAA	0.844	0.081	0.081	0.096
5-P	5.93	0.294	0.294	0.050
5-S	1.98	0.126	0.126	0.064
AP enzymatic assay				
AP	21.9	0.002	0.002	9.11×10^{-5}
AP-PAA	43.0	0.017	0.017	3.96×10^{-4}
5-P	12.9	0.020	0.020	1.55×10^{-3}
5-S	6.55	0.011	0.011	1.68×10^{-3}
Lip enzymatic assay				
Lip	0.373	0.005	0.005	0.013
Lip-PAA	0.122	0.002	0.002	0.016
5-P	0.133	0.001	0.001	0.007
5-S	0.070	0.001	0.001	0.014
HRP enzymatic assay				
HRP	0.107	0.034	0.034	0.318
HRP-PAA	0.461	0.142	0.142	0.308
5-P	0.796	0.123	0.123	0.155
5-S	0.330	0.032	0.032	0.097
LDH enzymatic assay				
LDH	0.198	0.072	0.072	0.364
LDH-PAA	0.073	0.007	0.007	0.096
5-P	0.196	0.007	0.007	0.036
5-S	0.196	0.006	0.006	0.031

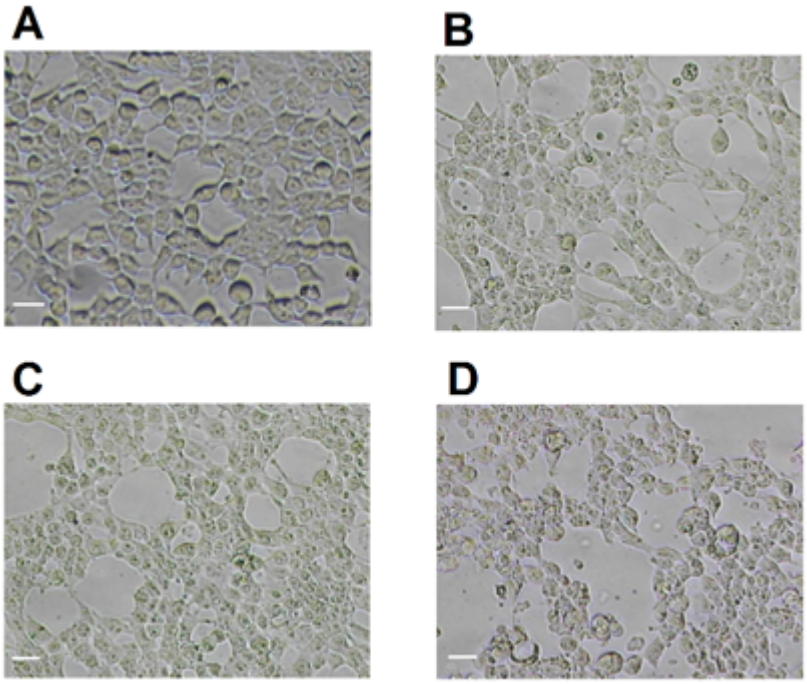

Figure S9: Significant dose-dependent cytotoxicity exhibited by 5-P conjugate co-incubated with HEK-293 cells at the following concentrations: A. 0 μM (control), (B) 240 nM, (C) 600 nM, (D) 1.2 μM.

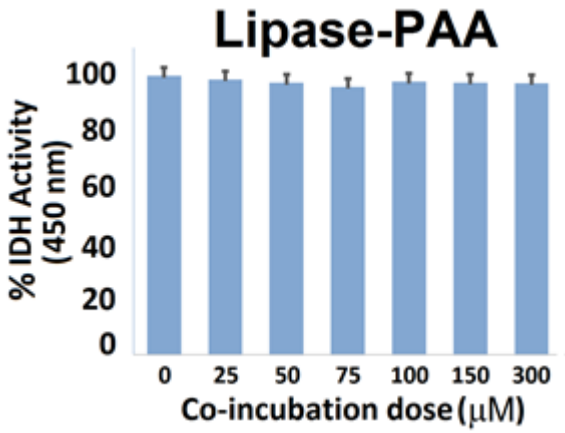

Figure S10: Dose-dependent in vitro cytotoxicity of Lipase-PAA conjugates as a function of intracellular dehydrogenase activity (IDH).

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